Limited proteolysis of rat liver nucleolin by endogenous proteases: effects of polyamines and histones

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Nucleolin is a major nucleolar phosphoprotein and is presumably involved in rDNA transcription and ribosome biosynthesis. This protein is known to be very labile and to be cleaved by endogenous proteases into many small peptides. We found that, when rat liver nucleolar suspension (Nu-1) or nucleolin-rich extract (Nu-2) was incubated under conventional conditions, polyamines and histones interacted with the nucleolin to lead to its preferential degradation to 60 kDa phosphopeptide (p60). The peptide p60 was identified as a peptide containing the N-terminal half of the nucleolin molecule, as judged from peptide-map analysis. Whereas spermine binding to the purified nucleolin was decreased by KCl concentrations above 50 mM, histones (H1, H2B and H3) were able to bind to the nucleolin in the presence of up to 300 mM KCl. A distinct difference between H1 and other histones was found in that H1 could produce p60 from nucleolin in both Nu-1 and Nu-2, whereas H2B and H3 stimulated the degradation of nucleolin to p60 only when Nu-2 was used for the source of nucleolin. A possible relationship between p60 formation and rRNA synthesis is discussed, but its exact role remains to be studied.

INTRODUCTION

Nucleolin (100–110 kDa; also called C23) is a ubiquitous phosphoprotein in eukaryotic cells; predominantly localized in the nucleolus. Numerous experiments using whole animals, intact cells, isolated nuclei and nucleoli have implicated nucleolin in the regulation of rDNA transcription (Bugler et al., 1982, 1987; Olson et al., 1983; Bouche et al., 1984; Escande et al., 1985; Gas et al., 1985; Jordan, 1987; Lapeyre et al., 1987; Erard et al., 1988; Egyhazi et al., 1988; Belenguer et al., 1989) and maturation of rRNA (Prestayko et al., 1974; Bourbon et al., 1983; Bouche et al., 1984; Herrera and Olson, 1986; Bugler et al., 1987), although its exact role remains to be determined. The amino acid sequence found previously indicates that the protein consists of three domains (Lapeyre et al., 1986, 1987; Bourbon et al., 1988; Schneider and Issinger, 1988; Olson et al., 1990; Bourbon and Amalric, 1990; Belenguer et al., 1990), the N-terminal domain possessing several concensus phosphorylation sites (Lapeyre et al., 1987; Schneider and Issinger, 1988; Belenguer et al., 1990). The phosphorylation of nucleolin is catalysed by casein kinase II (Caizergues et al., 1987; Pfaff and Anderer, 1988; Warrener and Petryshyn, 1991) and enhances the proteolytic cleavage of nucleolin (Warrener and Petryshyn, 1991).

We previously showed that testosterone and polyamines stimulate the phosphorylation of nucleolar proteins in rat ventral prostates and that spermine increases the degradation of nucleolin to a 59 kDa phosphopeptide by a serine-type protease (Suzuki et al., 1985). The presence of similar peptides in nucleoli was reported also during oogenesis and embryogenesis of *Xenopus laevis* (Caizergues-Ferrer et al., 1989). However, detailed studies were hampered because of the comparatively small size of rat prostates. Later, we found that phosphorylation of nucleolin as well as rRNA synthesis in liver was also controlled by a steroid, glucocorticoid, through a change in the activity of nucleolar casein kinase II (Suzuki et al., 1987, 1991). On the other hand, nucleolin was reported to bind to histone H1 at physiological ionic strength and induce chromatin decondensation (Erard et al., 1988). Thus the present investigation was undertaken to examine whether and how spermine and histone H1 enhance the limited proteolysis of nucleolin to 60 kDa phosphopeptide (p60) in liver nucleoli.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (4500 Ci/mmol) and [^{14}C]spermine tetrachloride (90 mCi/mmol) were purchased from ICN Biochemicals, Irvine, CA, U.S.A. and from CEA, France, respectively. Peroxidase-conjugated goat anti-rabbit IgG antiserum was from Seikagaku Kogyo Co., Tokyo, Japan. Anti-nucleolin antiserum was prepared as described by Suzuki et al. (1991).

Isolation of nucleoli and preparations of nucleolin-rich fraction (Nu-2) and purified nucleolin (Nu-3)

Nucleoli were isolated from livers of male rats (Wistar strain, 150–200 g) as described previously (Suzuki et al., 1987). From the nucleolar suspension (termed Nu-1), a nucleolin-rich fraction (termed here Nu-2) was prepared by the method of Bourbon et al. (1983). Briefly, the nucleoli were incubated at 4 °C for 30 min in the presence of 1 mM EDTA (pH 7.4), followed by centrifugation at 10000 *g* for 5 min. About 50% of the proteins in the supernatant was nucleolin, as judged by SDS/PAGE. For experiments of binding of spermine and histones to nucleolin, a more purified nucleic acid-free nucleolin fraction (termed Nu-3) was prepared as follows. From nucleoli isolated in the presence of 0.5 mM phenylmethanesulphonyl fluoride (PMSF), an Nu-2 fraction was obtained by extraction with 1 mM EDTA (pH 7.4) containing PMSF (0.5 mM) and leupeptin (20 μg/ml). After solid urea and RNAase were added to the Nu-2 fraction to final concentrations of 6 M and 10 μg/ml respectively, the mixture was incubated at 4 °C for 18 h. The mixture was then passed

Abbreviations used: NBS, *N*-bromosuccinimide; PBS, phosphate-buffered saline (10 mM sodium phosphate, pH 7.2, containing 0.15 M NaCl); PBS-T, PBS containing 0.05% Tween 20; p60, 60 kDa phosphoprotein; PMSF, phenylmethanesulphonyl fluoride.

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through a Sephadex G-25 column (PD-10) which was previously equilibrated with buffer M [20 mM Tris/HCl (pH 7.5) containing 0.5 mM PMSF and leupeptin (10 μg/ml)]. The flow-through fraction was applied to an anti-nucleolin antibody-affinity column prepared by coupling anti-nucleolin IgG to CNBr-activated Sepharose 4B (Pharmacia) in accordance with the manufacturer's instructions, and the column was washed with buffer M. Nucleolin was then eluted with buffer M containing 3 M KSCN and the eluate was passed through a PD-10 column (Pharmacia) by using buffer M. The flow-through fraction was concentrated and diafiltrated against buffer M, with a Centriflow apparatus (CF 25 membrane; Amicon). The resulting solution was termed Nu-3.

Phosphorylation and degradation during incubation of nucleoli and nucleolin with [γ-^{32}P]ATP, spermine and histones

Nu-1 fraction was suspended in 0.1 ml of 50 mM Tris/HCl (pH 7.5) containing 5 mM $MgCl_2$ and 10 μM [γ-^{32}P]ATP (50 μCi/ml) and the mixture (50–100 μg of protein/ml) was incubated at 37 °C for 10–120 min. When Nu-2 fraction was used instead of Nu-1 fraction, the fraction containing 10 μg of protein was incubated, in a total of 0.1 ml, in 50 mM Tris/HCl (pH 7.5) containing 6 mM $MgCl_2$, 0.05 mM EDTA and 10 μM [γ-^{32}P]ATP (50 μCi/ml). Spermine, histones or protease inhibitors were added to the mixture as described in the appropriate experiments. In experiments at low pH, 50 mM Tris/HCl (pH 7.5) was replaced with 50 mM Tris/acetate (pH 5.5), and [γ-^{32}P]ATP was removed. In this case, Nu-2 fraction was prepared from Nu-1 fraction labelled with ^{32}P for 10 min under the conditions described above.

SDS/PAGE and immunoblot analysis

Nu-1 or Nu-2 was labelled with ^{32}P and subjected to SDS/PAGE and transblotting as described previously (Suzuki et al., 1987). The proteins were then transferred to nitrocellulose membrane as described by Towbin et al. (1979) in a semi-dry blotting apparatus (Nihon Eido, Tokyo, Japan). The blotted membrane was then washed six times with PBS-T and incubated for 60 min in PBS containing 3% BSA. After several washes, the membrane was incubated with 1/2000-diluted anti-nucleolin antiserum or non-immune control serum. After six washes with PBS-T, the membrane was incubated for 1 h with peroxidase-conjugated anti-rabbit IgG antiserum. Unbound antibodies were washed out extensively with PBS-T and the blots were stained with 4-chloro-1-naphthol and H_2O_2.

Chemical cleavage of nucleolin with *N*-bromosuccinimide (NBS)

Chemical cleavage of nucleolin with NBS was carried out by the method of Rao et al. (1982). A mixture (400 ul) containing 50 mM Tris/HCl (pH 7.5), 6 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (1 μCi) and the Nu-2 fraction (30 μg of protein) was incubated at 37 °C for 10 min. After addition of 133 μl of acetic acid (final concn. 25%, v/v), 1 mg of solid NBS was dissolved in the mixture and the chemical reaction was carried out for 60 min at room temperature. The reaction mixture was then diluted 3-fold with water, and freeze-dried.

Digestion of nucleolin and its fragments with V8 protease and peptide mapping

Bands of nucleolin and its fragments on SDS/PAGE were cut out from the gels, and the peptides were digested with V8 protease (0.25 and 1.0 μg/sample well) during the second SDS/PAGE as described by Cleveland et al. (1977).

Binding of spermine to nucleolin

The incubation mixture contained, in a total volume of 200 μl, Nu-3 (10 μg of protein), 10 μM [^{14}C]spermine (90 mCi/mmol) and 20 mM Tris/HCl (pH 7.5). After incubation for 15 min at room temperature, the mixture was applied to a PD-10 column which was previously equilibrated with Tris/HCl buffer (pH 7.5), and washed with 5 ml of the same buffer. The buffer was then changed to 0.1 M HCl containing 0.5 M NaCl, and radioactivity was measured in each fraction (0.5 ml) obtained. In parallel experiments, 50 mM KCl or 1 mM spermine (non-radioactive) was added to the incubation mixture.

Binding of nucleolin to histones

Binding of nucleolin to histone H1 was examined with a H1-affinity column (Erard et al., 1988), and with nitrocellulose membranes bound to H1. The histone H1 column was prepared by coupling histone H1 with CNBr-activated Sepharose 4B and equilibrating with 20 mM Tris/HCl (pH 7.5) containing 1 mM EDTA, 1 mM β-mercaptoethanol, 0.5 mM PMSF, 30% glycerol and 50 mM KCl. The Nu-3 fraction phosphorylated as mentioned above was dialysed against the equilibrium buffer and applied to the column. After the column had been washed with 5 ml of the same buffer, nucleolin was eluted with a linear gradient of KCl (0.05–1.0 M). A portion of each fraction was subjected to SDS/PAGE, followed by Coomassie Blue staining. The remaining part of each fraction was used for measurement of ^{32}P radioactivity. The binding experiments with nitrocellulose membranes were carried out as follows. After histone H1 (2 μg/well) was spotted with a slot-blotter (Dassel, Germany) on to the nitrocellulose membrane, the membrane was incubated for 1 h at room temperature in the binding buffer (20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone and 0.2% BSA), and then for 3 h in the same buffer containing ^{32}P-labelled Nu-3 (1 μg of protein/slot), lysozyme (200 μg/well) and various concentrations of KCl (50–450 mM). Subsequently, the membrane was washed extensively with the binding buffer containing the corresponding concentrations of KCl, and subjected to autoradiography. The binding of H2B and H3 to nucleolin was carried out similarly.

Determination of protein, DNA and RNA contents

The protein concentration was determined by the dye-binding method described by Bradford (1976), with ovalbumin as the standard. The concentrations of DNA and RNA were determined by the methods of Burton (1956) and Schneider (1945) respectively.

RESULTS

Effects of polyamines on the degradation of nucleolar proteins by endogenous proteases

A nucleolar suspension (Nu-1) incubated for various time intervals with [γ-^{32}P]ATP in the presence (1 mM) or absence of spermine was subjected to SDS/PAGE. Figure 1(a) (protein staining) shows that the incubation caused the degradation of many peptides, especially peptides of 125, 100, 65 and 32 kDa (see the arrowheads), which was stimulated by 1 mM spermine. As seen from lane 1 of Figure 1(b) (autoradiogram), incubation for 10 min without polyamine brought about the

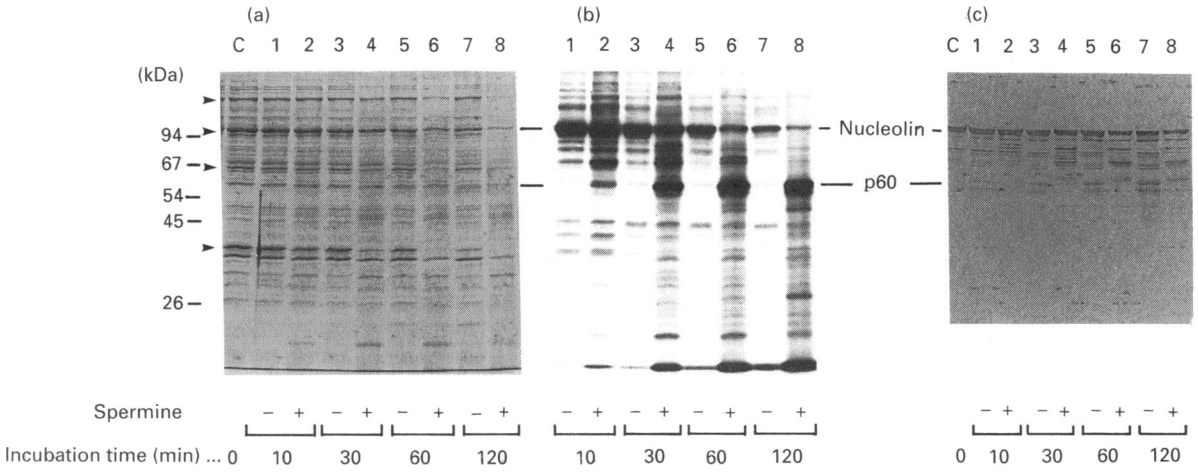

Figure 1 Degradation of nucleolar proteins during incubation of nucleolar suspension (Nu-1) in the presence and absence of spermine

Nucleolar suspension (Nu-1; 1 mg/ml) was incubated with [γ-^{32}P]ATP in Tris/HCl buffer (pH 7.5) at 37 °C for the period of time indicated in the presence (1 mM) or absence of spermine. Portions of the samples were then subjected to SDS/PAGE, followed by staining with Coomassie Brilliant Blue (**a**) and autoradiography (**b**). Other portions of the samples were separately subjected to SDS/PAGE and transferred to nitrocellulose membranes, followed by immunostaining with anti-nucleolin antiserum (**c**). Arrowheads indicate peptide bands which showed significant degradation. Sizes of markers are given in kDa. Lane C, control.

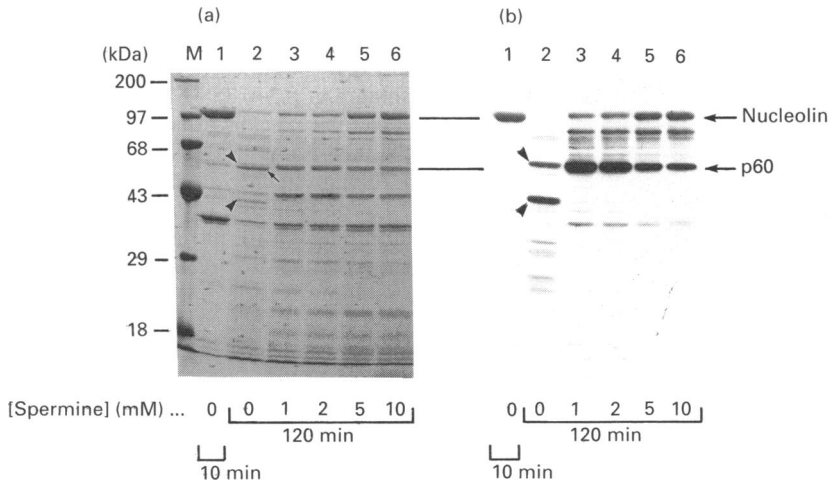

Figure 2 Effect of spermine on the proteolysis of nucleolin during incubation of Nu-2 fraction

Nucleolin-rich fraction (Nu-2) was prepared by extraction with 1 mM EDTA from Nu-1. The Nu-2 (0.1 mg/ml) was incubated at 37 °C with [γ-^{32}P]ATP for the time intervals indicated in the absence (lanes 1 and 2) or presence of spermine (lanes 3–6). At the end of incubation, sample was subjected to SDS/PAGE, followed by protein staining (**a**) and autoradiography (**b**). Arrowheads indicate phosphorylated 60 kDa (p60) and 42 kDa proteins; arrow indicates non-phosphorylated 59 kDa protein. M shows marker proteins.

phosphorylation of 11 peptides, one of which is the major peptide, nucleolin, 100–110 kDa in size. These peptides gradually disappeared after prolonged incubation even without spermine (see lanes 3, 5 and 7 of Figure 1b). When the incubation was performed in the presence of 1 mM spermine, enhancement of the phosphorylation of the 11 phosphopeptides, together with the appearance of new phosphopeptides including the 60 kDa peptide (p60), occurred. It is noteworthy that prolonged incubation with spermine diminished all bands except p60 (and several smaller faint peptides). It is likely that p60 is a degraded product of nucleolin, in view of the result of the immunoblot with anti-nucleolin antiserum (Figure 1c), which was taken from a parallel experiment to the sample treated similarly. The results were reproducible in two to four repeated tests, as in the other experiments described below.

The above experiments were carried out with nucleolar suspension (Nu-1). Since spermine affects solubilization of nucleolar proteins by changing the structure of the nucleoli, some of these accompanying proteins might affect the proteolysis of nucleolin. We have therefore attempted to study the effect of spermine with a nucleolin-enriched preparation (Nu-2). The preparation was obtained by treatment with 1 mM EDTA for nucleolar suspension (Nu-1) (Bourbon et al., 1983), and its nucleolin content actually reached about 50% of total proteins, another entity being the 37 kDa peptide (lane 1, Figure 2a). Incubation of Nu-2 without spermine under phosphorylation conditions for 10 min and 120 min clearly shows the phosphorylation of nucleolin (lane 1, Figure 2b) and of 60 and 42 kDa peptides (arrowheads, lane 2, Figure 2b) respectively. Incubation for 120 min with 1 mM spermine stimulated the degradation of nucleolin to p60 (lane 3

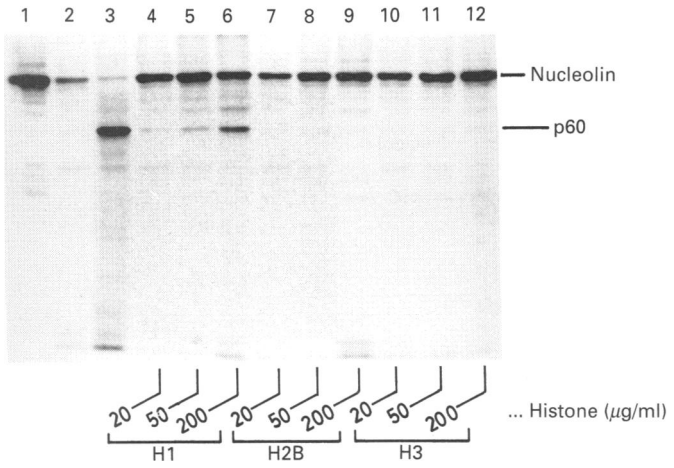

Figure 3 Effects of histones (H1, H2B and H3) on the degradation of nucleolin in the nucleolar suspension (Nu-1)

Nu-1 (1 mg/ml) was incubated for 120 min at 37 °C with [^{32}P]ATP and in the presence of H1 (lanes 4–6), H2B (lanes 7–9) and H3 (lanes 10–12) as indicated. As control experiments, the Nu-1 was incubated for 10 min (lane 1) or 120 min (lanes 2 and 3) without histones under phosphorylation conditions. Spermine (1 mM) was present in the incubation of lane 3.

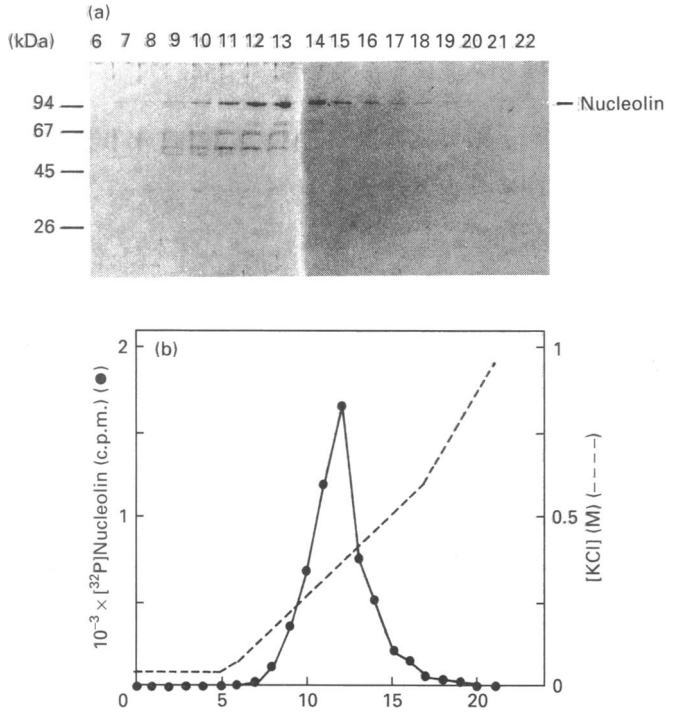

Figure 4 Binding of nucleolin to histone-H1 affinity column

Purified nucleolin fraction (Nu-3) was applied to a histone-H1 affinity column, and then eluted with a linear gradient of KCl (0.05–1.0 M). The eluate in each fraction was subjected to SDS/PAGE and Coomassie Blue staining (**a**) and counted for radioactivity (**b**).

of Figures 2a and 2b). The degradation of nucleolin was, however, diminished by using higher concentrations of spermine (lanes 4–6, Figures 2a and 2b). It was also noticed that incubation for 120 min in the absence of spermine produced a 59 kDa peptide (arrow, lane 2 of Figure 2a) which is likely different from p60, since it is not phosphorylated under our phosphorylation conditions.

The direct binding of nucleolin to spermine was examined as described in the Materials and methods section by using purified nucleolin (Nu-3; 95% pure, nucleic acid-free). From these experiments it was found that the binding really occurs under the conditions mentioned above, but is abolished in the presence of 50 mM KCl. Thus it is unlikely that the binding of nucleolin to spermine takes a role in the metabolism *in vivo*.

Effect of histones on the proteolysis of nucleolin by endogenous proteases

It was previously reported that the binding of histone H1 with nucleolin may participate in transcription of rDNA (Olson et al., 1983; Jordan, 1987; Erard et al., 1988; Zlatanova, 1990). We therefore examined whether histone H1 exerts any effect on the degradation of nucleolin under similar conditions. As with polyamines, the incubation of Nu-1 or Nu-2 with histone H1 of lower concentrations also caused the stimulation of limited proteolysis and stabilization of nucleolin. Then we compared the effects of three histones on the limited degradation of nucleolin. Figure 3 shows the results obtained when Nu-1 was used as the source of nucleolin. It is clear that the preferential degradation of nucleolin to p60 was found in the presence of H1, but not in the presence of H2B and H3. However, the degradation of nucleolin to p60 was observed also with H2B and H3 when Nu-2, instead of Nu-1, was incubated in these histones (results not shown).

The interaction of histone H1 with highly purified nucleolin (Nu-3) was studied with a histone H1 affinity column, as shown in Figure 4. Panel (b) indicates that nucleolin is eluted from the column at 0.3–0.35 M KCl. Panel (a) shows protein staining in each tube, revealing that the protein amounts are in parallel with the incorporation of ^{32}P.

Similar binding of Nu-3 with histones H2B and H3 was found by using H2B and H3 affinity columns (results not shown). The experiments with nitrocellulose membranes confirmed the similar binding of nucleolin to histones under physiological conditions (results not shown).

Characterization of the endogenous protease and its product (p60) from nucleolin

It was previously reported that nuclei contain two kinds of proteases, thiol-type and serine-type, whose activities were high at acidic and at neutral pH, respectively (Chong et al., 1974; Tsurugi and Ogata, 1980). In order to find which protease is responsible for the degradation of nucleolin, the effect of protease inhibitors on the degradation of nucleolin was examined. In Figure 5, ^{32}P-labelled Nu-2 fraction was incubated at pH 7.5 in the presence of leupeptin (lanes 2 and 3) or at pH 5.5 with PMSF (lanes 4 and 5) to inhibit thiol-type or serine-type protease, respectively, in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of spermine. Stabilization of nucleolin by spermine was observed under both acidic and neutral conditions. On the other hand, 42 kDa phosphoprotein, which was observed in the absence of spermine, and spermine-dependent p60 (lanes 2) appeared under neutral conditions. These results indicate that spermine interacts with nucleolin at lower pH as well as at neutral pH, and that degradation of nucleolin to p60 and 42 kDa phosphopeptides was catalysed by serine-type protease, which was still associated with nucleolin in the preparation in the presence of spermine. The results also showed that the degradation of nucleolin to 59 kDa peptide mentioned above (Figure 2) was not inhibited by PMSF and leupeptin, but was inhibited by spermine (see small arrows in Figure 5a-1).

The effects of protease inhibitors on the degradation of nucleolin were examined also in the presence of histone H1

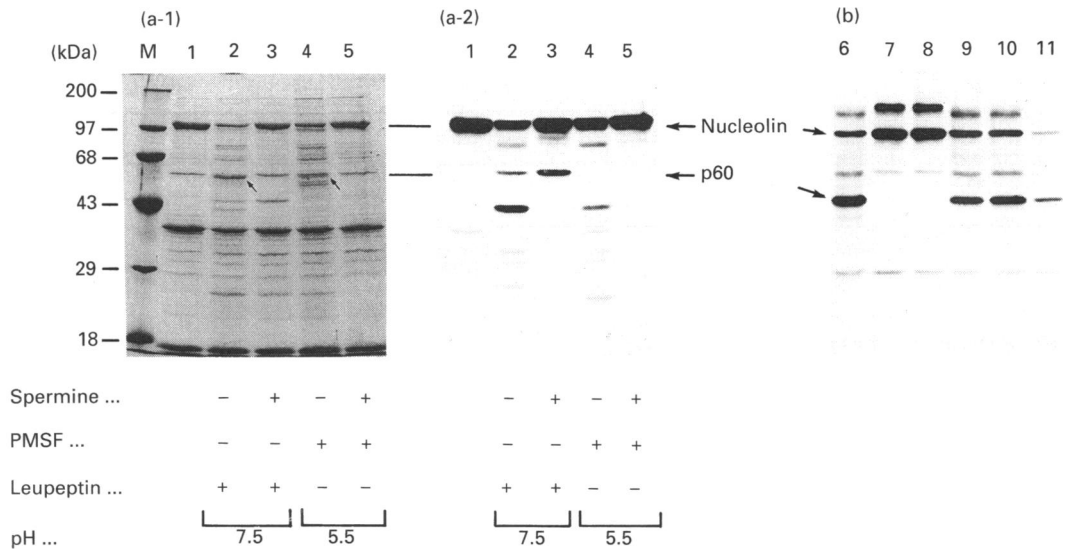

Figure 5 Characterization of proteases working on the proteolysis of nucleolin

(**a**) Influence of spermine and inhibitors. The ^{32}P-labelled Nu-2 fractions (100 μg/ml) were incubated for 120 min at 37 °C in Tris/HCl buffer (pH 7.5) containing leupeptin (40 μg/ml) (lanes 2 and 3) or Tris/acetate buffer (pH 5.5) containing PMSF (1 mM) (lanes 4 and 5), in the absence (lanes 2 and 4) or presence of 1 mM spermine (lanes 3 and 5), followed by SDS/PAGE (**a**-1) and autoradiography (**a**-2). As a control, the ^{32}P-labelled Nu-2 was subjected directly to SDS/PAGE (lane 1). (**b**) Influence of histone H1 and inhibitors. The ^{32}P-labelled Nu-2 was incubated for 120 min at 37 °C in Tris/HCl buffer (pH 7.5) with histone H1 (50 μg/ml) in the presence of 1 mM PMSF (lane 7), soybean trypsin inhibitor (100 μg/ml) (lane 8), antipain (200 μg/ml) (lane 9), leupeptin (40 μg/ml) or 0.1 mM p-chloromercuriphenylsulphonic acid (lane 11). Lane 6 is a control experiment which was carried out with H1 and without inhibitors. Autoradiography after SDS/PAGE was carried out as usual. M shows marker proteins.

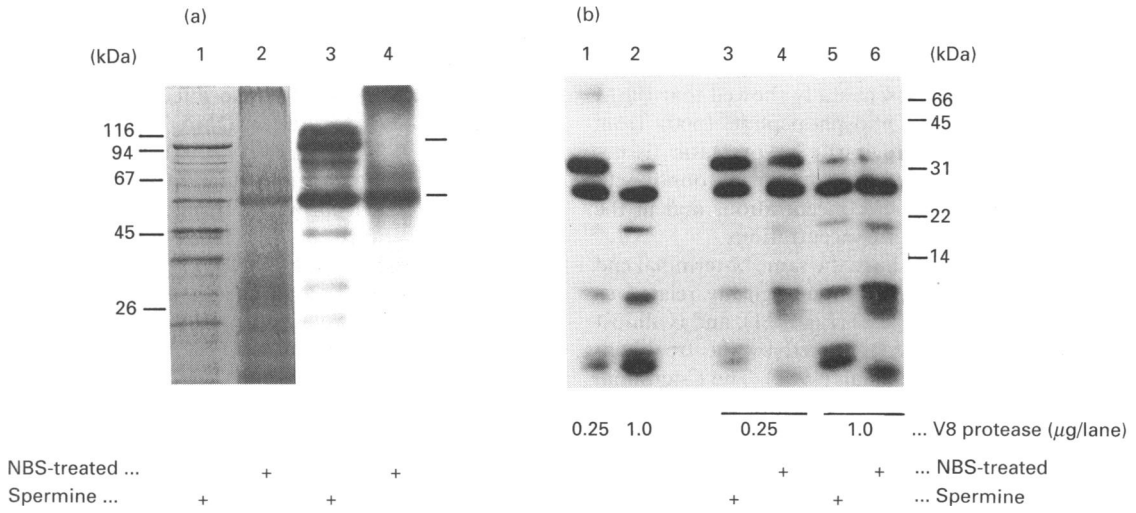

Figure 6 Comparison of fragments obtained from NBS-treated nucleolin with those from necleolin fragments produced by endogenous proteases

(**a**) Nu-2 fraction labelled with [γ-^{32}P]ATP was incubated for 120 min at 37 °C in the presence of 1 mM spermine (lanes 1 and 3) or for 60 min at room temperature with NBS (lanes 2 and 4), followed by SDS/PAGE and then Coomassie Blue staining (lanes 1 and 2) or autoradiography (lanes 3 and 4). (**b**) Nu-2 fraction labelled with [γ-^{32}P]ATP was treated as mentioned above, and the bands corresponding to p60 produced by endogenous proteases (lanes 3 and 5) and by NBS (lanes 4 and 6) were cut out from the gel, and peptide-mapping analysis using V8 protease was carried out as described in the Materials and methods section. As control experiments, the nucleolin band prepared from ^{32}P-labelled Nu-2 was treated with V8 protease, followed by SDS/PAGE (lanes 1 and 2). The amounts of V8 protease employed were 0.25 μg (lanes 1, 3 and 4) and 1.0 μg (lanes 2, 5 and 6).

(Figure 5b): H1-stimulated degradation of nucleolin to p60 is abolished by inhibitors of serine-type proteases (see lanes 7 and 8).

To determine the location of p60 in the parent molecule, the electrophoretic mobility and V8-protease-treatment pattern of p60 were compared with those of the N-terminal half of nucleolin, which was produced with NBS treatment, possessing all of the phosphorylation sites (Rao et al., 1982). From the results (Figure 6), it is likely that p60 is almost identical with the NBS-produced 60 kDa peptide of Rao et al. (1982).

DISCUSSION

Recent studies on the primary structure of nucleolin have indicated that the protein consists of three domains, as shown in Figure 7 (Lapeyre et al., 1986, 1987; Bourbon et al., 1988; Olson

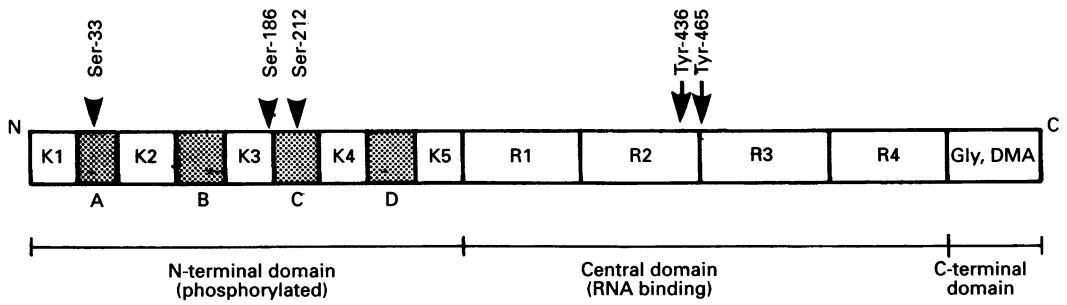

Figure 7 Schematic representation of nucleolin primary structure

The N-terminal one-third consists of acidic regions (A–D) and lysine-rich segments (K1–K5). The central domain is composed of four closely related 90-residue segments (R1–R4). The C-terminal domain contains approx. 60 residues. Arrowheads indicate the sites phosphorylated by casein kinase II, and arrows show the number in the sequence. DMA, dimethylarginine.

et al., 1990; Bourbon and Amalric, 1990). It is considered that the first domain, containing an N-terminal (about 300 residues), is elongated and binds to histone H1 with its acidic regions (A–D) and to DNA with its basic regions (K1–K5), whereas the central domain, composed of four closely related 90-residue sections, is a relatively compact unit interacting with RNA, and the C-terminal domain (approx. 60 residues, enriched in glycine and dimethylarginine) is of a non-globular conformation (Olson et al., 1990).

The N-terminal portion of nucleolin in which phosphorylation sites reside (Lapeyre et al., 1987; Caizergues-Ferrer et al., 1987) is known to be highly susceptible to proteolysis (Olson et al., 1990). Thus phosphorylated nucleolin (100–110 kDa) of rat liver was cleaved on incubation at 37 °C to 90 kDa, 80 kDa and especially 60 kDa phosphopeptides and then further to smaller peptides (Figures 1 and 2). The present study showed that 1 mM spermine protected the 60 kDa phosphopeptide (p60) from further degradation, as found previously in prostatic tissues (Suzuki et al., 1985). Similar actions were also found with spermidine and putrescine at higher concentrations and in the presence of histones of much lower concentrations.

The p60 peptide is considered to have the same N-terminal end as the parent protein, since it was immunologically related to nucleolin, having phosphorylation sites (Figure 1), and is almost identical with the 60 kDa peptide produced by the treatment with NBS (Figure 6) found by Rao et al. (1982). The *C*-terminal amino acid was not determined in both peptides. However, the treatment with NBS under their conditions is considered to cleave tyrosine bonds of nucleolin (Rao et al., 1982), and the most plausible end may be Tyr-436 or Tyr-465, on the basis of the molecular size of the peptide and the sequence of rat nucleolin (Bourbon and Amalric, 1990). These tyrosine residues are located in the area between R2 and R3 (arrows, Figure 7), which is known to be susceptible to trypsin and chymotrypsin (Olson et al., 1990). It is tempting to assume that the formation of p60 from nucleolin was derived by the attack of an endogenous protease on the exposed area of nucleolin, the other parts of which are protected by histones, DNA and RNA.

The protease responsible for the cleavage of liver nucleolin to p60 was found to be a serine-type enzyme (Figure 5), as in the prostate (Suzuki et al., 1985). In addition to the enzyme, liver seems to contain also a thiol-type protease, which produces a 59 kDa peptide with no phosphorylation site. It is not clear, however, whether this enzyme is related to the thiol-type protease in Chinese-hamster ovary cells reported previously by Bourbon et al. (1983), since no information was given about the phosphorylation of their product (60 kDa).

The physiological significance of proteolytic degradation of nucleolin to p60 in liver remains to be determined. The formation of smaller peptides (50 and 48 kDa) from nucleolin was found in *Xenopus laevis* oocytes from stages I–III and related to transcription of rDNA (Caizergues-Ferrer et al., 1989). In Chinese-hamster cells, the synthesis of pre-rRNA is inhibited by the protease inhibitor leupeptin, and this was also interpreted by the participation of a nucleolin maturation process in the rDNA transcription (Bouche et al., 1984). Although the present results were obtained in experiments *in vitro*, it is noteworthy that the preferential formation of p60 from nucleolin was observed on incubation with histone H1, but not H2B and H3, when Nu-1 was used as nucleolin source (Figure 3). Nu-1 (isolated nucleoli) are considered to reflect the state *in vivo* more than Nu-2, which was obtained after treatment with EDTA, being separated from nucleolar chromatin and associated proteins. Histone H1 is known to bind to linker DNA and inhibit transcription (Weintraub, 1984; Zlatanova, 1990). It was also reported that nucleolin is capable of inducing chromatin decondensation by displacing H1 from chromatin by interacting with the C-terminal part of H1 (Erard et al., 1988). On the other hand, the phosphorylation of nucleolin was catalysed by casein kinase II in many tissues (Caizergues-Ferrer et al., 1987; Suzuki et al., 1987; Saito et al., 1988; Schneider and Issinger, 1988), and that the phosphorylation of nucleolin in 3T3-F442A cells enhanced its proteolytic degradation to 30 kDa and 72 kDa peptides (Warrener and Petryshyn, 1991). The activities of casein kinase II and rRNA synthesis were found to be dependent on hormonal stimulus in hepatocytes (Suzuki et al., 1987) and lymphosarcoma P1798 (Suzuki et al., 1992) and on cell growth in HeLa cells (Schneider and Issinger, 1989). These facts raise the possibility that the phosphorylation of nucleolin and its preferential degradation to p60 or other smaller peptides may be something to do with pre-rRNA synthesis and maturation of rRNA. Further studies with intact cells are needed to inspect this hypothesis concerning the formation of the smaller peptides from nucleolin.

We are grateful to Ms. Aoi Uchida and Ms. Hiroko Okazaki for technical assistance. This work was supported in part by Grants-In-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and from the Hamaguchi Foundation for the Promotion of Biochemistry, Chiba, Japan.

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Received 28 April 1992/10 July 1992; accepted 6 August 1992